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According to their stainability with OsO, these ending-like elements seem to be classified into two categories; cholinergic ones containing ACh and SP and non-cholinergic ones containing Nor and 5HT. The former is lighter and lightly stained with OsO_4. (Author)

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THE ABSTRACT OF THE FINAL REPORT NO. 2

1. By ESR spectra and magnetic susceptibility measurement, the ferromagnetic behavior of liver ribosome, ribosomal RNA and thymus DNA was studied. The results obtained strongly suggested that RNA and DNA of high molecular weight are ferromagnetic in themselves and hence they would be so even in the ribosomes or nuclei.

2. In view of the fact that little attention has been paid to the brain microsomes, some studies on them were made. Using the method of isolating liver ribosome, we could found that brain ribosomes were also composed of 110s, 80s and 60s component. Electronmicroscopically, they appeared very similar to liver microsomes.

3. Using ultracentrifugation in the density gradient of sucrose subfractionation of brain homogenate was carried out. The mitochondrial as well as the microsome particles were found to contain some physiologically active substances such as ACh, SP, Nor and 5HT. Our electronmicroscopical observations strongly suggested, however, that these substances, which have been assumed as a candidate of central transmitter hitherto, were normally present in the particles similar to the isolated nerve ending described by de Robertis school. The activity of these substances recovered in the microsomal fraction seems to be attributed to the microvesicles (i.e., synaptic vesicles) originated from the disrupted endings.

4. According to their stainability with OsO_4, these ending-like elements seem to be classified into two categories; cholinergic ones containing ACh and SP and non-cholinergic ones containing Nor and 5HT. The former is lighter and lightly stained with OsO_4.

PHYSICO-CHEMICAL STUDIES

ON

THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES

The Final Report No. 2

Dr. Akira Inouye
Professor of Physiology
Department of Physiology
Faculty of Medicine
Kyoto University
Kyoto, Japan

CONTENTS PAGE

I. THE PURPOSE OF THIS INVESTIGATION

In the first year period of this contract, the physicochemical properties of liver ribosome and its RNA were chiefly studied. Because of the well-established method of isolating liver microsomes, ribosomes and RNA, most of studies on RNP, RNA or microsome have been made on liver, little attention being paid to those of brain. From the physiological point of view, however, the role played by brain microsomes appears not always to be identical with liver ones. Indeed, some active substances such as acetylcholine and choline esterase have been reported to be present in the microsomal particles of brain homogenates (1, 23), while some evidence for participation of brain RNA to learning was reported (13). In view of such an importance of microsomal particles in the brain function, therefore, our research project in this second year period was focused on the brain microsomal function.

The present report is mainly concerned with these problems:

1. Magnetochemical studies on the RNA of high molecular weight as a supplement to the studies in the previous year.

2. Investigation as to whether we can treat brain ribosome in the way similar to that of liver ribosome.

3. Studies on the subcellular unit (including microsome) of rabbit brain.

II. EXPERIMENTALS

1. Isolation of ribosome and RNA.

Liver ribosome and its RNA were isolated by the procedure same as that described in the previous report. The similar procedure was also applied to the brain ribosome with a slight modification; more concentrated desoxycholate solution (15 % instead of 5 % for liver ribosomes) was employed because of its high content of lipid.

2. Physicochemical measurement and chemical analyses of ribosomes.

Electrophoretic and ultracentrifugal analyses were made by the methods quite similar to those described in the previous report.

For analyses of RNA and protein, the conventional method of Littlefield (17) and Lowry (18) were also employed respectively.

3. Electronmicroscopy.

Electronmicroscopical observation of brain ribosomes were also made by the procedure for the liver one described in the previous report. Other specimens were examined by the conventional techniques.

4. Magnetochemical measurement on ribosome and RNA.

The static magnetic susceptibility, χ, was measured with an electromagnet (field strength 2×10^4 Gauss) and a hand-made magnetic balance of pendulum type which was consist of an analytical balance and an electronic recording device. The swing of the balance arm was transformed into electrical potential by a differential transformer and a suitable amplifier, which was fed into an automatic recording system. By means of the balance thus constructed, a considerably good signal; noise ratio at the gain of 1 dyne/20 cm deflection of recorder could be obtained at the room temperature (22 C).

For ESR studies, X-band spectrometer having low voltage type Klystron (Varian V-4500) or high voltage one (Tokyo Denki Seiki) was employed, their field modulation frequency being 400 and 120 cps respectively.

5. Homogenation and centrifugal fractionation of brain tissue.

Homogenation and subcellular fractionation of rabbit brain were usually made as described by Kataoka (16). The crude mitochondria were further subfractionated by centrifugation in the layered sucrose solution of 0.8 - 1.8 M; This stepwise density gradient was usually made up of 0.32, 0.8, 1.0 1.2, 1.4 and 1.8 M. In some experiments, we tried to apply the continuous density gradient; using an apparatus similar to that reported by Britten and Roberts (7), 0.8 M and 1.7 M sucrose solution were mixed under continuous stirring. One ml of crude mitochondria suspended in 0.8 M sucrose was layered on the top of this density gradient. Such a subfractionation was made by Hitachi 40P ultracentrifuge with a swing-bucket rotor similar to SW 39 of Spinco Model at 122000 × g for 30 min. The 5-7 subfractions were cut out by a tube slicer. After washing with 0.15 M sucrose, on each sample we made chemical and biological determinatio s as well as electronmicroscopical observation.

For subfractionation of crude microsomal fraction, stepwise density composed of 0.32, 0.55 and 0.8 M was used. In some experiments, a continuous gradient (0.32 - 0.8 M) was also used.

To obtain pure nuclei of brain tissue, rabbit brain was homogenated in 2.2 M sucrose solution. The homogenate was then centrifuged at 108000 × g for 60 min. The nuclei thus obtained was found to be nearly 100 % pure. But such a method provides too small amount of nuclei to isolate the nuclear ribosome. We could not yet find a suitable method of isolating pure nuclei which gives a suffieient yield to isolate ribosome. So we applied tentatively the following procedure: brain tissue was homogenated in 0.25 M sucrose, which was layered on 0.32 M sucrose in the centrifuge tube, and centrifuged at 900 × g for 10 min. The sediment suspended in 0.32 M sucrose was again layered on 0.8 M sucrose and centrifuged at 8000 g for 20 min. Increasing the sucrose concentration in the bottom of centrifuge tube to 1.2 and 1.4 M successively, such a centrifugation in the density gradient was repeated at 10000 g for 30 min. The nuclei thus obtained was of about 90 % purity by microscopical examination. This nuclei was used for the isolation of nuclear ribosomes.

6. Chemical and biological assay.

Activity of acetylcholine (ACh) was determined by bioassay on the frog's rectus muscle, that of serotonine (5HT) on the rat's fundus (25) after aceton extraction (2), that of substance P (SP) on the guinea pig ileum in the presence of atropine, pyribenzamine and 5HT (16).

Noradrenalin (Nor) was determined by spectrofluorometry (4), succinic dehydrogenase by Schneider-Potter's method (21), acid phosphatase by de Deuve's method (3).

III. RESULTS OBTAINED

1. Magnetochemical property of the ribosome and its ribonucleic acid.

a. ESR spectra of ribosome and RNA. It has been already reported (5, 6, 19, 22) that DNA of high molecular weight showed a broad ESR spectra similar to that of ferromagnetic substance. If such a ferromagnetic property of DNA is originated from its molecular structure, it could be expected that RNA of high molecular weight and ribosome containing it are also ferromagnetic. Hence we examined their magnetochemical property.

Indeed, liver ribosome and RNA of high molecular weight prepared from it showed a broad ESR spectra similar to that of DNA. To exclude the contribution of iron as a ferromagnetic impurity, these samples were dialysed for several hours in the presence of EDTA. Only the trace or none of iron was found in the RNA sample, while considerable amount of iron could be detected in the ribosome sample which was hardly eliminated with a mild procedure. Even after dialysis, RNA showed a broad ESR spectra, though it was weakened. Further experiments on the ribosome were abandoned because of its containing considerable ferromagnetic contaminant.

b. Static magnetic susceptibility of RNA. Such a ferromagnetic property of RNA was also observed by its χ measurement in spite of lacking in high sensitivity of our hand-made magnetic balance. It was highest at pH of about 8 and markedly diminished in the alkaline media (pH above 10). After hydrolysis, it became diamagnetic, its ferromagnetic behavior could not be recovered by neutralyzation of hydrolyzates.

c. Effect of magnetic field on the ribosomes. If the magnetic properties of RNA has a physiological significance in the role of ribosomes as a field of protein biosynthesis, strong magnetic field would affect the ribosomal activity of incorporating amino acids. Using liver ribosomes and those prepared from the reticulocytes of rabbits made anemic by phenylhydrazine administration, incorporation rate of C^{14}-leucine was examined under the field strength of 2×10^4 Gauss, the rate of incorporation decreased by about 15-20 %, while this magnetic field showed hardly any effect on the oxygen uptake of liver slice or blood cell suspension determined polarographically.

2. Microsomes and Ribosomes of Rabbit Brain.

a. Electronmicroscopical observation. When the microsomal fraction of rabbit brain was subfractionated by centrifugation in the density gradient, the lighter particles remaining in 0.32 M sucrose appeared to be mainly composed of small solid particles (200 - 500 Å), which are similar to free ribosomes. By means of spraying-shadowing method and negative staining method, we confirmed the presence of particles quite similar to purified liver ribosome in their apperance. Though we can not exclude the possibility that they are detached from the so-called rough-surfaced reticulum during the homogenation procedure, we scaresely found such free ribosomes in the liver microsomal fraction. It might be said, therefore, that in the brain cells free ribosomal particles are present. Ultracentrifugal analysis showed, however, that this subfraction was considerably contaminated with substances of high molecular weight, probably proteins.

With desoxycholate treatment, prufied ribosome could be obtained from this subfraction. Electronmicroscopical observation showed, however, that such a purification procedure rather causes considerable deterioration of ribosome: electronmicrographs of brain ribosome are not so sharp as those of liver ribosome reported in the previous report. Compared with those of crude lighter particulate subfraction, they were less sharp, a fact suggesting that our purification procedure was not adequate to isolate brain ribosome.

The heavier subfraction of the brain microsomes appeared to be chiefly composed of membranous structures and microvesicles. The former might be of endoplasmic reticulum origin (L1). The lightest particulate fraction isolated from the crude mitochondria also resembles this subfraction in its appearance. Microvesicles (500 - 2000 Å) found in these fractions may be disintegrated reticulum or the so-called synaptic vesicle described by Whittaker (27).

b. Ultracentrigugal analysis. It was already reported (12, 24) that the ribosomes isolated from Escheriolia Coli were composed of 100 s, 70 s, 50 s and 30 s particles. We also reported in the previous report that liver ribosomes were composed of 110 s, 80 s, 60 s and 40 s component. It would be expected from the similarity of brain ribosomes to liver ones in their electronmicrograph, that brain ribosomes were also composed of these unit members. Unfortunately, as stated above, our purified sample of brain ribosome was suffered from some deterioration and so isolation of each component was difficult. But ultracentrifugal pattern of our purified sample showed the main peak of 80 s component with a small peak of about 110 s and 60 s particles, a fact suggesting that they are very

similar to liver ribosomes. It sems necessary for more accurate analysis to prepare more intact sample of brain ribosome by overcoming technical difficulties.

c. The nuclear ribosomes. In view of the fact that nuclei play an important role as the very carrier of information in protein biosynthesis, investigation on the nuclear ribosomes seems very important. Quite recently, a few reports on them began to appear (15, 20, 26). We also tried to isolate nuclear ribosomes from brain. As the first step, preparation of pure nuclei was attempted as stated in the previous section. But the yield of nuclei is too small to use as the source of nuclear ribosomes. So we were compelled to use the nuclei sample somewhat contaminated with other cellular components. Even with this material, isolation of ribosomes was very difficult and unsatisfactory, our results obtained to date being only that the RNA-rich particulate suspension could be prepared and trace of 80 s component could be detected by ultracentrifugal analysis. Electronmicroscopical observation failed to prove the existence of particles of typical ribosomal structure. The further study will be made in the next year period.

3. Physiological characteristics of microsomal and mitochondrial particles.

a. Biological activity of subcellular units of rabbit brain. As stated above, the presence of choline esterase in the microsomal particles was already reported some investigators (1, 23). The first detailed report on the presence of a neuropharmacologically active substance, ACh, and 5HT in the particles lighter than the true mitochondria was that of Whittaker (27), who revealed by centrifugation in the density gradient that ACh was contained in a microvesicular fraction lying between the mitochondrial and microsomal one (so-called synaptic vesicle fraction). We confirmed his results on ACh but not on 5HT (16). In the course of these experiments, the microsomal particles also contained considerable amount of ACh, 5HT and substance P (SP), while they were detected in the heavier particles. Thence we felt the necessity of examining more thouroughly physiological distinction and/or significance of Whittaker's synaptic vesicle fraction and so-called brain microsomal particles.

Our results obtained by the centrifugal subfractionation of brain homogenates could be summarized as follows: Distribution of ACh and SP in the crude mitochondrial particles appears to run parallel with each other, their highest content being found in 1.0 M sucrose solution and only small amount of them in 1.4 M sucrose, while highest amount of noradrenalin (Nor) and 5HT was found in 1.25 - 1.4 M sucrose. The maximum activity of SDH, a typical mitochondrial enzyme was recovered in 1.2 M sucrose, while that of acid phosphatase, a typical lysosomal enzyme in 0.3 M sucrose. The microsomal particles, which were recovered in 0.32 M sucrose, also contained ACh, SP, and 5HT considerably, but only trace or non of SDH activity could be detected in them.

b. Electronmicroscopical observation. In the electronmicroscopical specimens prepared from ACh-rich 1.0 M subfraction and Nor-rich 1.25 M subfraction, the nerve ending-like elements reported by de Robertis group (7, 8) or by Gray and Whittaker (10) could be found besides typical mitochondria and other numerous vesicular structures. In 1.4 M fraction, aggregation of vesicular elements was marked, making their identification difficult.

The ending-like elements appear to be of two types, the one being densely stained with OsO_4, the other less densly stained. At a glance the former seemed to be found in the heavier particulate fraction. We tried to count such ending-like vesicles of dense and less dense type in the micrograph of 0.8 - 1.2 M subfractions obtained by the stepwise gradient, and we found fairly good parallelism between the ratios dense type: less dense type and Nor: ACh.

Subfraction	Dense type : less dense type	Nor : ACh
0.8 M	0.28	0.85
1.0 M	0.30	0.31
1.2 M	1.73	1.70

Some of such ending-like structures, especially less densely stained one, were found to enclose many microvesicles (300 - 800 Å), which are similar to synaptic vesicles described by Gray and Whittaker (10) or by de Robertis and his coworkers (7). Judging from their appearance, size and enclosed microvesicles, our ending-like structures could be said to correspond to the nerve endings described by these investigators.

In the crude microsomal fraction, in which activity of acetylcholine and other active substances were found, numerous microvesicles similar to such synaptic vesicles could be electron-microscopically observed besides larger vesicular structures (1000 - 3000 Å) and solid small particles (200 - 500 Å). Their nature has not yet been identified. But it seems highly probable that microvesicles are synaptic vesicles disrupted from the endings and small particles are free ribosomes.

IV. COMMENT ON THE RESULTS

1. On the magnetochemical property of RNA.

As stated above, DNA was reported to have a broad ESR spectra (6, 19, 22). Using calf thymus nuclei and DNA extracted from them, we confirmed it, but the protein isolated from thymus nuclei never showed such spectra. So our ESR observation techniques could be said to have no serious technical error compared with those of previous investigators. On the other hand, we could observe at the same time that ribosomal RNA of liver as well as liver ribosomes themselves provide ESR spectra similar to that of DNA. It might be said, therefore, that RNA, a substance similar to DNA in its structure, has ESR spectra quite similar to that of DNA and it would be ferromagnetic if the latter be so. Ferromagnetic behavior of RNA proved by our hand-made magnetic balance provides a further support for this view. Hence behavior of ribosome would not be unexpected one, even though iron contamination in our ribosome sample could not be eliminated sufficiently.

If the magnetic property of our RNA sample results from the trace of iron contained, it should originate from a specific structure of iron stabilized on the nucleic acid molecules, for instance colloidal micells of iron hydroxide, disappearance of ferromagnetic behavior after hydrolysis of RNA being attributed to breakdown of such a micellar structure. At any rate, the peculiar magnetic property of RNA and DNA might be said to originate from their specific molecular or micellar structure, not from their ferrogagnetic contaminant.

2. On the brain ribosomes.

Because of high content of lipid material in the brain tissue, isolation of brain ribosome was very difficult conventional DOC treatment was not always successfully applied and so we tried to use more concentrated DOC (15 %). As stated above, however, even such a modification was not yet so satisfactory: considerable deterioration was observed electronmicroscopically. The lighter subfraction separated from the crude microsomal fraction and not purified with DOC treatment appear to provide for rather typical image of ribosomes, a fact sug suggesting that free ribosomal particles are present in the microsomal fraction. In fact, ultracentrifugal pttern of this crude fraction gave a peak corresponding to 80 s particle, though very small and somewhat hard to identify.

Ultracentrifugal patterns of our purified brain ribosomes

were also unsatisfactory, only suggesting the presence of 80 s, 110 s and 60 s component. At present it should be concluded, therefore, that purification procedure of liver ribosome may be unapplicable. Since any systematic investigation on the brain ribosomes has not yet been reported hitherto, further detailed experiments seem derirable.

Our attempt to isolate the nuclearribosome were also failed because of two low yield of pure nuclei and difficulty of siolating brain ribosomes. Probably the brain tissue may be not adequate as the material for preparing nuclear ribosome, thymus gland tissue used by Mirsky group (20) seems better. The further trial to isolate nuclear ribosomes and to compare them with cytoplasmic ones will be made in the next year period.

3. Physiological characteristics of microsomal and mitochondrial particles.

In contrast with rather homogeneous liver tissue, brain is morphologically and functionally very heterogeneous. So the studies on brain homogenates harbours many complicated problems in analysing the results obtained. But it is evident from works of previous investigators or our own, that brain subcellular particles contain substances specific for neuronal function. In this respect, microsomal and mitochondrial particulate fraction of brain homogenate are distinctly diff different from those of liver.

Our aubcellular fractionation studies by centrifugation in the density gradient clearly demonstrated that acetylcholine, substance P, noradrenalin and serotonine are contained in the subcellular unit of density different from that of typical mitochondria or lysosomes. Electronmicroscopical examination should the presence of nerve ending-like structures in the particulate subfractions rich in these active substances. It seems worthy to note that they are classified into two types, densely stained and less densely stained with osmic acid. Counting such ending-like elements in the micrographs of each subfraction, we found that the ratio, dense type: less dense type, runs parallel with that of noradrenalin (or serotonine) : acetylcholine (or substance P) in its general trend. Though thin-sectioned electronmicroscopical specimen can not always considered as providing for a sample of average composition, such a fact seems to suggest that noradrenalin and serotonine are chiefly contained in the densely stained elements, acetylcholine and substance P in the less densely stained one.

Gray and Whittaker (10) and de Robertis and his co-workers (7) already demonstrated very fine electronmicrograph of nerve endings isolated by centrifugation in a density gradient. Recently, de Robertis group (8) reported that the cholinergic and non-cholinergic nerve ending could be separated from each other in the brain homogenates, the former being lighter than the latter and characterized by the presence of aacetylcholine. Some of our electronmicrographs showed that our ending-like structures also enclose small vesicles (so-called synaptic vesicles) as pinched off nerve ending of Gray-Whittaker or de Robertis. It might be said, therefore, that our densly stained elements correspond to the non-cholinergic ending of de Robertis group, while the less densly stained are to the cholinergic one.

These nerve endings are to have been pinched off from the axons or synaptic sites during homogenation and remained without further disruption. But it would be naturally expected that some of endings are further disintegrated by mechanical effect of homogenization, their synaptic vesicles being liberated into surrounding media. Taking their size (500 - 800 A) into account, liberated synaptic vesicles should be recovered in the microsomal fraction, when they were subjected to the centrifugation in the density gradient. The appearance of acetylcholine and other active substances in the microsomal fraction would be thus explained by disrupture of nerve endings and liberation of synaptic vesicles. Indeed, microvesicles similar to such a synaptic vesicle could be found in the crude microsomal fraction as already demonstrated in the pioneer work of Whittaker (27).

Of course, it would not be appropriate that all the biochemically or physiologically active substances found in the microsomal fraction originate from the microvesicles disrupted from endings or other heavier, larger particles. Our preliminary observations suggest that choline esterase may be of endoplasmic reticular origin, while monoamine oxydase activity found in the crude microsomal fraction of brain appears to be different from that in the crude mitochondrial one. Further studies are needed to clarify the plysiological significance of the microsomal particles of brain.

At any rate, it seems worthy to note here, that the subcellular particles containing substances which have been assumed as candidate of central transmitter hitherto could be classified into two kinds.

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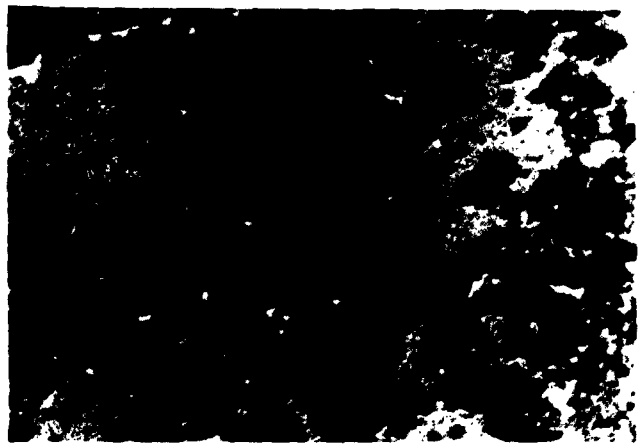
APPENDIXES

1



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